Origin and Destiny of Cholesterol in the Animal Organism.
Part XIII—*On the Autolysis of Liver and Spleen.*

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In Part XII of this series (1921) the comparison was made of the intake and output of cholesterol in normal adults on a known diet and over periods of six days, and an average daily loss of 0·3 grm. of cholesterol was noted. The conclusion drawn was that there must be some organ in the body capable of synthesising cholesterol. A similar view has been put forward by Grigaut (1913), who expressed the opinion that this synthesis is the function of the suprarenal glands. It seemed likely that the study of the autolysis of various tissues under aseptic conditions might throw some light on this question.

A number of observations bearing on this subject are described in the literature, some undertaken with the object of finding evidence of the presence of enzymes capable of hydrolysing cholesterol esters, others with the object of ascertaining whether destruction or synthesis of cholesterol took place on autolysis. The results are very conflicting.

In 1896, Carbonne, and again Waldvogel, in 1906, claimed to have obtained a large increase in cholesterol when lecithin was autolysed with sterile liver juice. Craven Moore (1907), however, was unable to confirm this, and described one experiment in which 600 grm. of human liver, containing 0·037 per cent. of cholesterol were autolysed aseptically for 42 days at 37° C., and at the end were found to contain 0·038 per cent. Corper (1912) also failed to find any marked change in the cholesterol of dog spleen on short autolysis.

Kondo (1910) attempted to decide the question of the presence of enzymes capable of hydrolysing cholesterol esters in blood and tissues by the determination of the acetyl values of the ether extracts, but his conclusions were vitiated by the presence in addition to cholesterol of other acetylisable substances.

In 1912, Schültze repeated these experiments, using the digitonin method for estimation of cholesterol and its esters. He found that in autolysis of human blood and horse blood no hydrolysis of cholesterol esters took place.

Rabbit's liver gave negative results, but in the case of horse liver a marked hydrolysis took place. In experiments with mixtures of blood and liver-juice, both in the case of horse and cow, a more or less complete hydrolysis took place, but not with serum and liver-juice. Schültze made no comment on the constancy or otherwise of the total cholesterol on autolysis, but as far as we can ascertain from his protocols, any variation between the values of the fresh and autolysed tissues were within the probable errors of experiment.

Cytronberg (1912) continued the work of Schültze and showed that the cholesterase is contained in the blood cells and not in the plasma. Howard Mueller (1916) however was unable to confirm Cytronberg's work.

In 1920, T. E. Abelous and L. C. Soula found that spleen pulp on autolysis at 37° showed a marked increase in total cholesterol. Some of their results are given in the following table:—

Percentage of Cholesterol in Spleen Pulp.

Animal.	Fresh.	After 24 hours autolysis.	48 hours.	6 days.	10 days.
Dog	0·231	0·930	—	—	—
Calf	0·500	0·580	0·680	0·399	0·150
"	0·350	0·450	0·150	—	—
"	0·078	0·459	—	—	—
Cow	0·317	0·570	0·415	—	—

Initially, it will be noticed, they found a rise followed on prolonged autolysis by a fall. They also found that these effects increased with rise of temperature up to 45° C.

In one experiment the addition of a minute amount of cholalic acid produced a still more marked synthesis of cholesterol.

Animal.	Initial content.	48 hours.	48 hours (with 0·05 grm. cholalic acid added).
Calf	0·500	0·68	1·11

They also investigated other organs and concluded generally that nervous tissue and liver also have the power of synthesising cholesterol, but in less degree than spleen. Other tissues on autolysis show a destruction of cholesterol.

The authors give no details of their methods of extraction and analysis, but obviously their results, if correct, are of very great importance.

We determined, therefore, to study the autolysis of various organs, and in this paper give an account of our experiments on liver and spleen.

Method.

The whole fresh organ, freed as completely as possible from adhering tissue and fat, was finely minced in a mincing machine, pulped in a mortar, and divided into the required number of approximately equal portions, which were then accurately weighed. One portion was analysed at once and the others mixed, in some cases, with sufficient toluene, and in other cases with a 2 per cent. solution of sodium fluoride to prevent bacterial growth. They were then placed in sterile flasks in the incubator at 37° C. and allowed to autolyse for definite periods. The autolysed tissue was not examined bacteriologically, but the smell indicated that no appreciable putrefaction had taken place.

Extraction of Fat.

This is the most important stage of the process, and one in which, in our experience, error is most likely to occur. The pulped tissue—fresh or autolysed—was mixed with excess of alcohol and the fluid portion then drained off. The solid matter was placed in a paper thimble and extracted with alcohol for several days, the material being taken out at intervals and re-ground. Finally the extraction was completed by means of ether.

The alcoholic fluids were evaporated and the residue thoroughly extracted with ether. The ethereal extract was added to the above and made to known volume. An aliquot portion was evaporated to obtain the total ether extract, and, when desired, to estimate the free cholesterol.

The extraction was carried out in a flask or matrass with a very long wide neck. The paper thimble was placed in an ordinary Soxhlet tube with syphon, suspended by a platinum wire in the neck of the flask. Above this was suspended a closely wound glass spiral tube through which cold water circulated, and which served as a very efficient condenser. By varying the height of the Soxhlet tube and the condenser the apparatus could be adapted for use with various solvents, and the temperature of the actual extracting solvent could be varied within considerable limits. Another important advantage was that all corks and joints were dispensed with, and the apparatus was easy to manipulate and clean.

Hydrolysis of the Fat.

The fat was hydrolysed in ethereal solution with a large excess of an alcoholic solution of sodium ethoxide, as described in previous papers, but as the esters of cholesterol are said to be difficult to hydrolyse in the cold by this method, the mixture was refluxed for 4 or 5 hours on the water-bath, and then allowed to stand 24 hours. The soaps were filtered and well washed with ether, or, if too large for this, extracted in the apparatus described above. The ethereal solution of unsaponifiable matter was then thoroughly washed free from alcohol and traces of soap, made to known volume, and an aliquot portion evaporated and weighed.

Estimation of Sterols Precipitable by Digitonin.

This estimation was carried out by the method described by Fraser and Gardner (1910) on a suitable portion of the solution.

Most of the experiments were carried out on human liver and spleen, obtained from cases of healthy adults killed suddenly by accidents. In these cases it was not possible to obtain the material until some 24 hours after death, during which time *post-mortem* changes might have taken place, with possible destruction of the enzymes responsible for the changes under investigation. Control experiments were therefore made on liver and spleen obtained from oxen killed at the slaughter-house by the Kosher method. The material thus obtained was treated within 2 or, at most, 3 hours after death. In one experiment pure cholalic acid was added to the autolysing tissue, to test the statement of Abelous and Soula that the addition of a small quantity of this substance would markedly increase the synthesis of cholesterol.

Our results are given in the following Tables—(I) Liver, (II) Spleen.

Discussion of Results.

In all these experiments, such aliquot portions were taken for analysis as would give between 0·18 and 0·25 grm. of digitonide for weighing. It was not possible to use larger quantities than this owing to the scarcity of digitonin, but in our experience these quantities are quite sufficient to give reliable results, other things being equal.

When we consider the large and complex series of operations necessary in carrying out these experiments the errors must be considerable, however great the care taken. We think that errors creep in mainly in the extraction process. In the Tables we give the sterol results to the third place of decimals, which would be justifiable if we were dealing with pure sterols. The relatively minute errors inherent in the digitonin method itself are, of course, magnified in calculating the quantities actually weighed to percentage of the original material taken for autolysis and extraction. In our opinion, second place may be regarded, however, as approximately correct.

A careful consideration of the figures indicates no evidence whatever of either synthesis, or destruction of cholesterol, during autolysis, and as far as

Table I.—Liver.

Source.	Weight of original organ taken for experiment.	Pre-servative used.	State of tissue.	Duration of autolysis.	Temperature of autolysis. ° C.	Ether extract.	Unsap. matter.	Sterol by digitonin.		Results.
1. Human (accident)	570	Toluene	Fresh from P.M.	—	—	3·18	0·29	0·146	Difference −0·007	
	570	,,	Autolysed	14 days	37	2·45	0·29	0·139		
2. Human (accident)	530	,,	Fresh from P.M.	—	—	6·95	0·63	0·268	Difference +0·017	
	530	,,	Autolysed	30 days	37	6·26	0·57	0·285		
3. Human (accident)	232·2	,,	Fresh from P.M.	—	—	6·37	0·79	0·257	Deviation from mean value 0·289	−0·032
	250·2	,,	Autolysed	24 hours	37	6·85	0·77	0·251		−0·038
	205·5	,,	,,	48 ,,	37	6·40	0·98	0·344		+0·055
	207·7	,,	,,	7 days	37	6·93	0·79	0·304		+0·015
4. Human (accident)	154·4	2 per cent. solution of sodium fluoride	Fresh from P.M.	—	—	4·78	0·64	0·295	Deviation from mean value 0·289	+0·006
	154·3	,,	Autolysed	1 day	37	4·67	0·63	0·292		+0·003
	161·9	,,	,,	6 days	37	4·82	0·77	0·281		−0·008
5. Ox liver (Kosher)	157·1	,,	Fresh from slaughter-house	—	—	6·89	0·59	0·284	Difference +0·013	
	155·3	,,	Autolysed	24 hours	37	6·70	0·65	0·297		

Table II.—Spleen.

Source.	Weight of original organ taken for experiment.	Pre-servative used.	State of tissue.	Duration of autolysis.	Tem-perature of autolysis. ° C.	Ether extract.	Unsap. matter.	Sterol free of digitonin.	Results.
1. Human (same subject as liver 3)	15·27	Toluene	Fresh from P.M.	—	37	2·31	1·43	0·366 Deviation from mean value 0·364	+0·002
	14·18	”	Autolysed	24 hours	37	3·46	1·47	0·353	−0·011
	17·70	”	”	48 ”	37	2·87	1·36	0·352	−0·012
	14·43	”	”	144 ”	37	3·43	1·31	0·385	+0·021
2. Human (same as liver 4)	29·80	2 per cent. sodium fluoride sol.	Fresh from P.M.	—	37	2·63	1·08	0·275 Deviation from mean value 0·296	−0·021
	29·95	”	Autolysed	24 hours	37	2·69	0·97	0·320	+0·024
	32·43	”	”	144 ”	37	2·16	0·99	0·304	+0·008
3. Human........	27·69	Toluene	Fresh from P.M.	—	37	2·23	0·95	0·428 Difference	−0·036
	23·35	”	Autolysed	24 hours	37	2·27	1·09	0·392	
4. Ox (Kosher)	140·3	2 per cent. sodium fluoride	Fresh from slaughter-house	—	37	2·63	0·63	0·447 Deviation from mean value 0·446	+0·001
	125·9	”	Autolysed	24 hours	37	2·07	0·72	0·439	−0·007
	174·6	”	Autolysed, with addition of 0·7 gr. of cholalic acid	48 ”	37	2·29	0·80	0·453	+0·007

autolytic experiments afford evidence on the point, these organs are not concerned with the synthesis of cholesterol in the organism.

In column 8, we give the percentages of unsaponifiable matter. These figures are probably a little too high, owing to the presence of traces of resins which we have shown (1921, Gardner and Fox) are produced by the action of alcohol on alkali during hydrolysis, but it will be noticed that the total unsaponifiable matter is two to three times the weight of sterol precipitated by digitonin. The composition of this unprecipitable portion is under investigation. With the Burchardt-Liebermann reagent this substance gives a reddish brown colour changing to green, in a manner similar to that of the portion of the unsaponifiable matter of fæces not precipitated by digitonin.

Estimations were also made of the free and ester cholesterol present in the fresh and autolysed tissue, but we do not give the figures at this stage of the inquiry, because we think that the question of the presence of ferments capable of hydrolysing or synthesising sterol esters, is better attacked by investigating the effects of tissue extract on pure cholesterol esters. We may say, however, that our results are in general agreement with those of J. H. Schültze, previously mentioned.

Conclusion.

As far as autolytic experiments are concerned, there is no evidence that either liver or spleen are the organs which have to do with the synthesis or change of cholesterol, as maintained by Abelous and Soula.

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